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(54) **ANALYSE DE L'ACTIVITE ENZYMATIQUE UTILISANT DES  
SUBSTRATS IMMOBILISES, MARQUES PAR  
FLUORESCENCE**

(54) **ANALYSIS OF ENZYME ACTIVITY USING IMMOBILIZED  
FLUORESCENCE-LABELED SUBSTRATES**

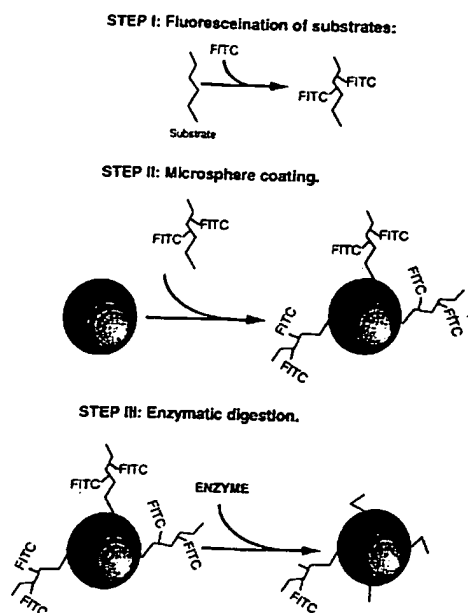


Figure 1 of 7

(57) Le précis n'est pas disponible en ce moment.

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Industrie Canada Industry Canada

## ANALYSIS OF ENZYME ACTIVITY USING IMMOBILIZED FLUORESCENCE-LABELED SUBSTRATES

### FIELD OF THE INVENTION

The present invention relates to a novel method of assaying enzymatic activity using optical detection methods. In particular, this method involves analysis of enzymatic activity using flow cytometry and immobilized fluorescent-labeled substrates.

### BACKGROUND OF THE INVENTION

Enzymes are proteins produced in a cell that are capable of greatly accelerating the chemical reaction of a substance (the substrate) for which they are often specific. They are divided into six main classes. Class 1 contains the oxidoreductases which catalyze the transfer of electrons; Class 2 comprises the transferases which catalyze group-transfer reactions; the hydrolases comprise Class 3 enzymes which transfer functional groups to water, resulting in the hydrolytic cleavage of bonds; Class 4 enzymes comprise the lysaes which catalyze the addition of groups to double bonds or the reverse; Class 5 contains the isomerases which transfer groups within molecules to yield isomeric forms; and, the ligases comprising Class 6, catalyze the formation of C-C, C-S, C-O, and C-N bonds by condensation reactions coupled to ATP cleavage.

Not only are enzymes thought of in terms of the types of reactions they catalyze, but many are thought of as being paired-up with a somewhat specific substrate. For example, a DNAase which degrades DNA can be conceptually paired with its substrate, DNA. In fact, many enzymes have been named by adding the suffix *-ase* to the name of their substrate. Thus urease catalyzes hydrolysis of urea and arginase catalyzes the hydrolysis of arginine. Though, other enzymes such as pepsin and trypsin have been given names not based on their substrate.

Enzyme assays typically measure the amount of enzymatic activity to determine the quantity of enzyme present and/or its level of activity. Some enzymatic activities are more amenable to the development of rapid, sensitive and reproducible assays than are others. Moreover, given the importance of enzymatic analysis in research and medicine, there is a constant need for improvement and advances in the field.

For example, in the field of medicine, detection of enzymatic activity in biological and chemical samples is useful for obtaining information about metabolism, diseases state, the identity of microorganisms, or the success of genetic manipulations. In such situations, in order to detect a number of diseases, samples of a body fluid, such as blood or spinal fluid, can be taken from a patient and tested to determine the presence of certain enzymes known to be present only during or after the occurrence of a specific disease.

Flow cytometry is a useful technique for identifying the presence of certain analytes or particles of interest in a sample, enumerating those particles and, in some instances, providing a sorting capability so as to be able to collect those particles of interest. (See McHugh *In* Darzynkiewicz, Robinson, and Crissman (eds.) *Methods in Cell Biology* (New York: Academic Press, 1994) 42:575-595).

Flow cytometry apparatuses rely upon the flow of particles in a liquid flow stream in order to determine one or more characteristics of the particles under investigation. In a typical flow cytometry apparatus, a fluid sample containing particles is directed through the flow cytometry apparatus in a rapidly moving liquid stream so that each particle passes serially, and substantially one at a time, through a sensing region. A focused light beam illuminates the particles in this region, and the instrument measures optical interactions of the light with each moving particle; for example, multiple wavelength absorption, scatter as a function of angle, and fluorescence as a function of either wavelength or polarization may be measured. After particle analysis is performed by the flow cytometry apparatus, those particles that

have been identified as having the desired properties may be sorted if the apparatus has been designed with such capability.

Representative flow cytometry apparatuses are described in U.S. Patent Nos. 3,826,364 and 4,284,412, and in the publication by Herzenberg *et al.*, (1976) *Sci. Am.* 234(3):108.

5 The use of colloidal particles and magnetic particles to bind a compound has long been known and used in industrial and laboratory procedures. For example, cross linked polystyrene-divinylbenzene beads, among the earliest and most widely used particles, have been used in organic synthesis, catalysis and the biotechnical arts, especially immunology. In combination with the appropriate reagents, the particles have been used to remove specific  
10 cells from a sample containing a plurality of cell types or to enhance the results of instrumental biomedical assays. Unless specified otherwise, the terms "particles", "spheroids", "spheres", "microspheres" and "beads" as used herein, are interchangeable.

Microspheres can be coated with different capture reagents or substrates that react with specific analytes in a sample. The fluorescence associated with each microsphere class can  
15 be quantitated in order to assay for different analytes in a sample. The flow cytometer can accurately detect different classes of microspheres based upon a physical characteristic such as size or color. The use of different microsphere classes, each coated with a different capture reagent, allows for the rapid and simultaneous detection of multiple analytes. This provides the potential to perform multiple assays in the same reaction mixture reducing cost  
20 and hands-on time, as well as generating results using the same method between analytes.

Microspheres have been used with a variety of capture reagents, including antigens (from infectious agents, cell surfaces, or other soluble proteins) to capture antibodies, antibodies to capture soluble antigens, receptors to capture immunoglobulins, oligonucleotides to capture products from the polymerase chain reaction, and proteins for competitive  
25 immunoassays with soluble protein or DNA. Usually after the reaction of the test sample

with the microspheres, a fluorescent detection reagent is added to the microspheres and allowed to react. The microsphere classes are then analyzed using flow cytometry and the different classes separated by size or color. Each microsphere class can then be analyzed independently: the fluorescence associated with each microsphere class is quantitated and used to indicate the presence or absence of the test substance. This method has been used to detect and separate antigens and antibodies in biological samples (U.K. Patent No. 1,561,042).

Flow cytometry has been used as a method of detecting antibodies to specific enzymes in biological samples. As an example, an immunoassay has been developed that uses pyruvate dehydrogenase enzyme complex as a specific antigen for diagnosis of primary biliary cirrhosis (PBC) (Elkhalifa *et al.*, (1992) *Am. J. Clin. Pathol.* 97(2):202-208). In this assay, pyruvate dehydrogenase enzyme complex was attached to polystyrene microbeads, incubated with sera from PBC patients, incubated with a fluorescein isothiocyanate conjugated goat anti-human immunoglobulin, then analyzed by flow cytometry. Flow cytometry and monoclonal antibodies have also been used in the detection of the human enzyme monocytic lysozyme in patients suffering from acute myeloid leukemia (Leculier *et al.*, (1992) *Blood* 79(3):760-764). These assays, however, do not measure enzymatic activity.

Flow cytometry has also been used to detect enzyme activity in cells. For example, fluorescent substrates have been used to determine beta-galactosidase activity in viable gram-negative bacteria (Flovins *et al.*, (1994) *Applied and Environmental Microbiology* 60(12):4638-4641); to detect enzymatic activity in microbial colonies (Sahar *et al.*, (1994) *Cytometry* 15(3):213-221); and to measure cellular dehydrogenase activity (Severin and Seidler (1992) *Cytometry* 13(3):322-326). These assays measure *in vivo* enzymatic activity using flow cytometry and fluorescently-labeled substrates that are permeable to cytoplasmic membranes.

Flow cytometry has also been used to show an augmentation of cell surface expression of MMPs (Leppert *et al.*, (1995) *FASEB J.* 9(14):1473-1481).

5 This review of enzyme assays currently available indicates that a need remains for a direct method of determining net enzymatic activity that is sensitive, reproducible, easy to use, and suitable for large-scale use. It is therefore an object of the present invention to provide an easy, reliable assay to determine qualitative and quantitative measurements of enzyme activity in biological samples on a routine basis.

10 This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties in this application.

## SUMMARY OF THE INVENTION

15 The problems noted above have been overcome by the use of a new method that allows qualitative and quantitative measuring of specific enzymatic activity in a sample. This method is comprised of the following steps:

- a. select an appropriate enzyme/substrate pair;
- b. labeling enzymatic substrate with fluorochrome;
- c. immobilizing the labeled substrate on a solid support;
- 20 d. adding a test sample to a portion of the immobilized labeled substrate under such conditions as to allow enzymatic digestion of the labeled substrate;

- e. washing the immobilized substrate;
- f. passing samples and controls through a flow cytometer that is capable of sensing the presence or absence of the label and that is also capable of identifying and/or quantifying any particular group of particles to which the labeling substance may be attached; and
- 5 g. identifying the amount of net enzyme activities in the test sample based on the measured signals.

In one embodiment of the present invention, a number of groups of microspheres are employed, with each group containing microspheres of a particular size range. Each group of microspheres is coated with a different enzyme substrate.

- 10 Another embodiment of the present invention involves using microspheres of the same size, but each is labeled with a separate photodetector; for example, colors could be used.

- In one embodiment of this invention, the substrate gelatin, labeled with fluorescein isothiocyanate, is immobilized on polystyrene microspheres and added to biological samples. The sample is then passed through a flow cytometer, and net gelatinase-B activity is  
15 determined.

Various other objects and advantages of the present invention will become apparent from the detailed description of the invention.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1. Fluorescence-Activated Substrate Conversion (FASC).

Figure 2. Analysis of substrate conversion.

Figure 3. Comparison of relative fluorescence of uncoated microspheres (A) and microspheres coated with FITC-labeled gelatin (B). On the left, forward-light scatter (FS) vs side scatter (SS) histograms of the respective microsphere preparations are shown.

5 Figure 4. Specificity of the enzymatic degradation of FITC-labeled gelatin. FITC-labeled gelatin (A) and casein (B) were coated on microspheres and incubated with 200 ng of purified gelatinase B for 16 h at 37°C. The histograms shown are representative of at least ten independent experiments with five different microsphere preparations for each substrate. Similar results were obtained with different FITC:protein ratios and different concentrations  
10 of substrates during coating.

Figure 5. Inhibition of gelatin degradation by blocking with monoclonal antibody. FITC-labeled gelatin-coated microspheres were incubated with 200 ng of gelatinase B in the presence of various amounts of inhibitory monoclonal antibody specific for gelatinase B. The final volume of the reaction was 100 µl. The results were expressed as the percentage  
15 of gelatin degraded in the presence of the blocking monoclonal antibody. Controls included microspheres without gelatinase B ( $MCF 239.4 \pm 1.3$ ), and microspheres with gelatinase B without monoclonal antibody ( $MCF: 21.0 \pm 2.4$ ). Maximal degradation in the absence of blocking monoclonal antibody was calculated as  $91.2 \pm 1.0\%$ .

20 Figure 6. Inhibition of gelatin degradation in the presence of low molecular weight inhibitors. FITC-labeled gelatin-coated microspheres were incubated with 200 ng of gelatinase B in the presence of the indicated concentrations of inhibitors. Inhibitors were preincubated for 15 min. at room temperature with gelatinase B prior to addition of microspheres. The enzymatic reaction was carried out at 37°C for 16 h. The results are expressed as the MCF of the microsphere populations obtained after treatment and are  
25 representative of two independent experiments.



Figure 7. Sensitivity of the assay. Five different microsphere preparations were obtained by varying the FITC concentrations (from 2.0  $\mu$ M to 1.25 mM) during fluoresceination of gelatin substrate. (A): fluorescence of the five microsphere preparations obtained. The five right end peaks correspond to five-fold increases in FITC concentrations. The left end peak corresponds to uncoated microspheres. (B) and (C): degradation of FITC-labeled gelatin using the lowest (B) and the highest (C) fluorescent microsphere preparations, as shown in (A). The results are representative of two independent experiments.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention resides in the discovery that net enzymatic activity can be quantitatively measured by monitoring the enzymatic degradation of labeled substrates immobilized on a solid support using optical detection methods.

Briefly, this technique entails the selection of an appropriate enzyme/substrate pair. A fluorophore is attached to the substrate, which is then immobilized on a solid support such as a microsphere. A test sample is added to a portion of the immobilized labeled substrate under such conditions as to allow enzymatic digestion of the labeled substrate, which is then washed. Finally, the immobilized substrate and controls are passed through a flow cytometer and the amount of enzyme activity in the test samples (eg. Net enzyme activity) and controls are measured.

In an alternative embodiment, the reaction can be stopped at a particular point in the digestion and the enzyme activity determined for that time point.

The method of the present invention will have many research applications and in particular will have applications for diagnostic and industrial use. As an example, this method will be useful for monitoring disease states in which enzymatic degradation of biological substrates is involved. In particular, this assay will be useful for monitoring enzyme activity in various

forms of arthritis, autoimmune diseases, and cancer metastasis. This assay will also be useful in the development of new enzymatic inhibitors for therapeutic uses; for example, it can be used to develop anti-metastatic and anti-inflammatory reagents for new therapeutic approaches designed to block tumor cell dissemination and ECM degeneration during inflammation.

This method can be used for routine screening procedures during quality control assays for recombinant and naturally occurring enzymes. As well, this method will be useful in the study of the regulation of enzymatic activity such as mechanisms of inhibition and activation of enzymatic products. It can also be used for the identification and characterization of new enzymatic substrates.

In certain applications, the method of the present invention constitutes a significant improvement over current methods of enzymatic analysis. It is characterized by high specificity, sensitivity, and reproducibility. One of the advantages of the present invention is its ability to measure net enzymatic activity. Many biological samples contain enzyme inhibitors. It is important to determine the net activity of enzymes in the presence of these inhibitors since biological activity often depends on the ratio of free active protein to inactive protein. The methods of the present invention allow the determination of net enzyme activity whereas other assays may not.

The method can also take advantage of the rapidity and reproducibility of laser flow cytometric analysis. The assay can be automated to require a minimum of handling, and can thus be applied to large-scale screenings of antagonist reagents or biological samples for diagnostic use. Routine screening procedures could also take advantage of flow cytometers equipped with an autoloader. Up to 300 samples can be analyzed per hour with minimal handling of samples. Flow cytometry also allows for the rapid and simultaneous detection of multiple analytes. This provides the potential to perform multiple assays in the same

reaction mixture reducing cost and hands-on time as well as generating results using the same method between analytes.

5 In a preferred embodiment, fluorescence-activated substrate conversion (FASC) is used to take advantage of the high sensitivity obtained by fluorescence-activated signals (using a 488 nm laser excitation wavelength). FASC is characterized by its high specificity, sensitivity, and reproducibility. It is also environmentally safe. In most cases, the signal-to-noise ratio between autofluorescent microspheres and those coated with the FITC-labeled substrate was near 500. This allows for accurate measurements of enzyme activity in the presence of chemical or biological inhibitors.

10 The steps of this invention and its principles are demonstrated using Gelatinase B, a metalloproteinase as an example. In particular, the examples presented herein, demonstrate that this method is useful for studying the regulation of gelatinase activity, for developing new anti-metastatic agents against MMPs, and for monitoring the activity of other proteolytic enzymes.

15 ***A Working Example: Gelatinase B/Gelatin***

20 Gelatinase B/gelatin has been selected as an enzyme/substrate pair to demonstrate effectiveness of the method of this invention because matrix metalloproteinases (MPP) play a crucial role in a variety of pathological conditions, such as joint degeneration in rheumatoid arthritis, demyelination in multiple sclerosis, and dissemination of malignant tumors. This example demonstrates that the rapid, sensitive, and reproducible assay of this invention can measure the net biological activity present in samples. Thus, a preferred embodiment of this invention is described for gelatinase B to demonstrate the use of flow cytometry in a powerful novel enzymatic assay method for the measurement, on a routine basis, of the net activity resulting from the balance between the gelatinase B activity and the presence of

natural inhibitors, as well as for the identification of new metalloproteinase inhibitors that could suppress the excessive proteolytic activity which characterizes many disease processes.

Gelatinase B (Class 3, E.C. 3.4.24.35) is a member of the MMP family of proteinases (MMP-9) and expresses a high degree of homology between species (Masure *et al.*, (1993) *Euro. J. Biochem.* 218:129-141; Tanaka *et al.*, (1993) *Biochem. Biophys. Res. Com.* 190:732-740). Accumulating evidence demonstrates a causal relationship between gelatinase B activity and the invasive behavior of tumor cell lines. Gelatinase expression by cancer cell lines has been correlated with the invasive and metastatic potential of these cells *in vivo* (Bernhard *et al.*, (1990) *Cancer Res.* 50:3872-3877; Bernhard *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:4293-4297; Ura *et al.*, (1989) *Cancer Res.* 49:4615-4621).

*In vivo*, the activity of MMPs is regulated by a family of natural inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). These compounds form a tight stoichiometric noncovalent complex with the activated MMPs and block their proteolytic activity (Woessner (1991) *FASEB J.* 5:2145-2154). As an example, administration of recombinant TIMPs to mice inhibit colonization of ras-transfected malignant cells (Alvarez *et al.*, (1990) *J. Natl. Cancer Inst.* 82:589-595), while suppression of TIMP activity by antisense technology enhances the invasive phenotype (Khokhe, *et al.*, (1989) *Science* 243:947-950). Development of specific inhibitors of MMP and gelatinase activity is of crucial importance in new therapeutic approaches designed to block tumor cell dissemination and ECM degeneration during inflammation. Key to developing such inhibitors is the need for an assay method that can measure, on a routine basis, the net activity resulting from the balance between gelatinase B activity and the presence of inhibitors.

Matrix metalloproteinases (MMPs) are thought to play a crucial role in extracellular matrix (ECM) degradation. The integrity of connective tissues is determined by the delicate balance between the synthesis and reabsorption of ECM molecules. Breakdown of this balance is associated with a variety of pathological conditions, such as joint degeneration in rheumatoid

arthritis, demyelination in multiple sclerosis, and dissemination of malignant tumors (Liotta *et al.*, (1991) *Cell* 64:327-336; McCachren (1991) *Arthritis. Rheum.* 34:1085-1093; Opdenakker and Van Damme (1992) *Cytokine* 4:251-258; and Opdenakker and Van Damme (1994) *Immunol. Today* 15:103-107.). Their ability to degrade collagen and other components of the ECM allows circulating leukocytes and tumor cells to progress through the basement membrane underlying the blood vessel wall, and invade the surrounding tissue. MMP activity is closely regulated by cell activation, and has absolute requirements of zinc and calcium for catalytic activity and structural stability (Woessner (1991) *FASEB J.* 5:2145-2154).

Given the important roles of MMP proteinases, one skilled in the art could easily appreciate the utility of a method of routine enzyme analysis in the field of medicine and research. A comparison can be made with previously used techniques, as there are several assays that have been developed to measure MMP activity (for a review of these methods, see Murphy and Crabbe *In Barrett (ed.) Methods in Enzymology. Proteolytic Enzymes: Aspartic Acid and Metallopeptidases* (New York: Academic Press, 1995) 248:470.). The gelatinolytic assay is based on the degradation of radiolabeled type I collagen. Although this method is relatively sensitive, it requires the use of radiolabeled specific substrates. Another widely-used technique is the zymography assay. In this assay, MMP activity is detected by the presence of negatively-stained bands following electrophoresis in gelatin-impregnated SDS polyacrylamide gels. The zymography assay is a sensitive and quantitative method for the detection of various MMPs in biological samples; nonetheless, it is labor intensive and has a low dynamic range. Zymography, moreover, is not suitable to measure the intrinsic net activity in biological samples because SDS dissociates MMP-TIMP complexes and activates latent enzyme forms; thus, zymography cannot be used to measure MMP activity in the presence of TIMPs. This is particularly important since matrix degradation ultimately depends on the ratio of free active gelatinase to latent proenzyme or TIMP-complexed forms.

A microtiterplate assay has been developed recently (Pacmen *et al.*, (1996) *Biochem. Pharm.* 52:105-111). This assay provides measurement of net biological enzymatic activity of MMP, does not require a radioisotope safety environment, and could be used efficiently for routine measurement of inhibitory activity of MMP; however, it is not likely to be highly efficient as a diagnostic test since the incubation times are long and the sensitivity is much lower than that obtained by standard zymography and radio-labeled substrate assays. The methods of the present invention allow the determination of net enzyme activity whereas other assays may not. For example, the standard zymography assay may detect gelatinase B activity in certain cell supernatants, whereas the present invention does not. This is because zymography detects both active and latent forms of the enzyme and dissociates gelatinase-TIMP complexes. The assay of the present invention, however, measures the net activity of gelatinase B in biological samples.

Thus, the gelatinase B example also demonstrates the need for improved, sensitive and rapid assays for routine diagnostic and research testing, particularly in the field of medicine. Also, this example also demonstrates how the use of fluorescence-emitting labels enables greater sensitivity and more rapid rates of assay than existing techniques.

#### **Selection of Enzyme/Substrate Pairs**

Although a preferred embodiment of this invention has been generated using the proteolytic enzyme, gelatinase B, this invention is particularly adaptable to hydrolases (Class 3), transferases (Class 2) and ligases (Class 6) enzymes. Within Class 3, the following enzymes are particularly adaptable to this method of this invention including: 1) the *esterases*, including exo- and endonucleases active on ribo- and deoxyribonucleic acids (e.g., DNase, RNAase, restriction enzymes), carboxylic ester hydrolases (e.g., phospholipase A1), etc.; 2) *glycosidases*, including hydrolyzing glycosyl compounds such as xylanase, insulinase, lysozyme and hyaluronidase; and 3) *peptidases* (synonymous of peptide hydrolases)

including exo-(amino- and carboxypeptidases and endopeptidases (proteinases such as gelatinases).

Any enzyme/substrate pair could potentially work in this invention if the functional group on the substrate is one that is labelable by the fluorophore, and removable by the action of the enzyme.

The enzyme to be assayed is paired with an appropriate natural or synthetic substrate, which is labeled with a fluorochrome. Examples of suitable pairs of enzyme and substrate are gelatinase B/gelatin; DNase/DNA; RNase/RNA; amylase/starch; various glycosidases/their polysaccharide substrates; peptidases/polypeptides. Other substrates that could be employed include MHC class I heavy chain (Demaria, S. et al., (1994) J. Biol. Chem. 269:6689-694), the folate receptor (Elwood, P.C. et al., (1991) J. Biol. Chem. 266: 2346-2353; the tumor necrosis factor-alpha precursor (Gearing A.J., et al., (1994) Nature 370:555-557; McGeehan, B.M. et al., (1994) Nature 368:558-560), and the fas ligand (Tanaka, M., et al., (1996) Nature Medicine 2: 317-322).

As shown in the following example, this method is demonstrated with gelatin, an enzymatic substrate for the enzyme gelatinase-B. In some cases, members of the metalloproteinase family can degrade *in vitro* other components of the ECM, including collagens, fibronectin, laminin, elastin, proteoglycans, and entactin (Matrisian, L.M. (1992) BioEssays 14:455-463) as well as synthetic decapeptides with a sequence identical to that derived from soluble  $\beta$ -amyloid sequence isolated from Alzheimer's disease patients (Miyazaki *et al.*, (1993) Nature 362:839-841). These are all examples of potential substrates for detecting MMP activity.

Ligases (e.g., peptide synthase, DNA and RNA ligases) which catalyze the joining of two molecules (an acceptor and a donor) with concomitant hydrolysis of the pyrophosphate bond in ATP, can be assayed using this method by immobilizing the unlabeled acceptor on microspheres. Measurement of ligase activity is obtained by the increased fluorescence of

the microspheres following ligation of the fluorochrome-labeled donor compound to the immobilized acceptor. In the case of transferases, enzymatic activity would be measured by monitoring the removal of the fluorochrome-labeled group from the donor compound immobilized on the microspheres.

## 5      **Generating Fluorescence Labeled Substrates**

10      It will be appreciated that any fluorescence-emitting detectable label may be used. Fluorescent substances used for labeling proteins are well known in the art. In one embodiment of this invention, substrates are labeled with fluorochromes. There are many constraints on the choice of fluorescer. One constraint is the absorption and emission characteristics of the fluorescer, since any materials in the sample under test will fluoresce and interfere with an accurate determination of the fluorescence of the label. This phenomenon is called autofluorescence or background fluorescence. Another consideration is the ability to conjugate the fluorescer to substrate and the effect of this conjugation on both the fluorescer and the substrate. A third consideration is the quantum efficiency of the  
15      fluorescer; this should be high for sensitive detection. A fourth consideration is the light absorbing capability or extinction coefficient of the fluorescer, which should be as large as possible. The choice of fluorescent reagent depends upon the assay configuration, reagent availability, and excitation/emission possibilities in the flow cytometer.

20      Fluorescent markers which are available include fluorescein isothiocyanate, Texas Red, phycobiliproteins, allophycocyanin, cyanine derivatives, and rhodamine. Rhodamine, a conventional red fluorescent label, has proved to be less effective. Texas Red is a useful labeling reagent that can be excited at 578 nm and fluoresces maximally at 610 nm. Phycobiliproteins, such as phycoerythrin, have a high extinction coefficient and high quantum yield. Cyanine dyes are described in U.S. Patent No. 5,486,616.



In a preferred embodiment, fluorescein isothiocyanate (FITC) was chosen as a fluorochrome for the following practical and theoretical reasons: 1) FITC is a small molecule; thus it minimizes the steric hindrance around putative cleavage sites; 2) it is easily conjugated to substrates; and 3) it has spectral properties compatible with most flow cytometers. Generally, FITC has been used most often because of its widespread availability in a variety of conjugates, its ease of excitation at 488 nm with argon ion lasers, and the availability of appropriate optical filters to detect emission at 525 nm. Although other fluorochromes, such as phycoerythrin or allophycocyanin have a better quantum yield than FITC, they may cause significant steric hindrance because of their high molecular weight. Texas Red or AMCA, which have low molecular weights and are easily conjugated to proteins, would probably be viable alternatives, although they require additional equipment to obtain adequate excitation.

The protein substrates of interest are dissolved in carbonate buffer (pH 9.2) to a final concentration of 2 mg/ml. Fluorescein isothiocyanate (FITC; Sigma; dissolved in MDSO at 5 mg/ml) is added to the appropriate concentrations. Labeling is carried out for 24 h at 4°C. Free FITC molecules are removed by chromatography on PD-10 columns (Pharmacia, Uppsala, Sweden) using PBS, pH 7.4 as eluent buffer.

#### **Immobilization of Labeled Substrates**

The solid support used in the claimed methods can be of variable but limited dimensions, generally ranging from 0.5 to 100 micrometers in diameter (most preferably 0.5 to 50 micrometers) and made of any substance provided that an enzymatic substrate can be either adsorbed onto or covalently bound to its surface. The substrate may be porous or hollow, or solid and non-porous. For reasons of cost, availability, and uniform size and shape, polymeric materials are preferred. Such polymers include polystyrene, polystyrene-divinylbenzene, polymethacrylate and polyphenylene oxide. Polystyrene and polystyrene latex supports are optimum because of their availability as various sized microspheres or

beads, inexpensiveness, compatibility with most biological systems, and familiarity to those skilled in the art. The polymeric substrate may contain amine-reactive surface functional groups; for example, aldehydes, aldehyde/sulfate, carboxylic acids and esters, and tosyl groups.

5 In a preferred embodiment, labeled substrate is immobilized on polystyrene microspheres. The use of polystyrene allows efficient noncovalent adsorption of most proteins. Although noncovalent adsorption to polystyrene is based only on electrostatic interactions and/or van der Waals forces, this coating is stable for months, provided the microspheres are kept in the dark at 4°C with 0.05% sodium azide as a preservative.

10 Smaller proteins, such as synthetic peptides, often do not attach well to polystyrene; in these cases, attachment to polystyrene may be achieved using covalent coupling techniques or an intermediate reagent (McHugh (1994) *supra*).

There are other techniques for attaching substrates to microspheres that are well known in the art.

15 In a preferred embodiment, polystyrene microspheres of 15 µm diameter are used to allow for maximal available surface in order to capture the greatest amount of fluorescent substrate and to generate an optimal signal-to-noise ratio.

20 Polystyrene microspheres of 15.5 µm (± 1.919) diameter (Polysciences, Warrington, PA) are incubated for 2 h at 37°C with FITC-conjugated substrates (1 mg/ml in PBS, pH 7.4) to allow noncovalent adsorption of the fluoresceinated substrates to the surface of the microspheres. The microspheres are then washed twice in phosphate buffer (pH 7.4) containing 0.5% BSA and 0.05% sodium azide (PBA). Microspheres are kept at 4°C in PBA (10<sup>6</sup> beads/ml) in the dark and resuspended by gentle vortexing before use.

The number of microspheres per reaction mixture may be varied. Enough microspheres must be collected to allow gating of single populations or to separate distinct microsphere classes and to produce an accurate fluorescent peak for measurement (McHugh (1994) *supra*).

#### **Enzymatic Digestion of Substrate:**

- 5 Incubation with the enzyme of interest is carried out for variable periods of time, usually from 60 to 90 minutes, under the appropriate conditions.

10 In the present invention, enzymatic activity is determined by a change in the signal of label as detected by a flow cytometer. It is understood that the present method may use many different types of flow cytometry or flow fluorometric apparatuses, whether measuring light scatter, particle volume, fluorescence, combinations of the foregoing or other optical, electrical or acoustical parameters for the identification, quantification, or classification of enzymatic activity in a sample fluid medium.

15 In a preferred embodiment of the present invention, the optical detection device is a laser flow cytometer with standard optics for collection of fluorescent signals. Although the assay has been developed on a Coulter XL-MCL flow cytometer equipped with an air-cooled argon laser emitting at 488 nm, analysis can be carried out with other commercially available bench-top flow cytometers found in most university hospitals.

20 The light source in the flow cytometer used in the present invention is not limited to the afore-mentioned argon ion laser; any other light source can be employed, such as a mercury arc lamp, axenon arc lamp, a He-Cd laser, a He-Ne laser, or a Krypton ion laser.

The assay in its present form requires minimal flow cytometer capabilities. It can be clearly upgraded through multicolor analyzes to allow simultaneous measurements of multiple

substrate degradation using different fluorochromes. Alternatively, simultaneous enzymatic degradation can also be monitored using microspheres with multiple diameters. In this case, a FS-SS histogram can be used to distinguish between different microsphere populations coated with fluorochrome-labeled substrates.

## 5      **Analysis of Samples and Controls**

10      Samples and controls are passed through the flow cytometer as illustrated in Figure 2. In certain instances the microspheres or other solid support will require a reading. The undigested labeled substrate coated on the microsphere or other solid support will also be read, as will the enzyme-digested sample. For a net enzyme analysis, the enzyme-digested sample will be allowed to digest to completion. Time course studies can be performed at samples arrested at the time point of interest. Someone skilled in the art will be able to determine the appropriate controls and time points appropriate to the design of the experiment.

15      The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

## 20      **EXAMPLE I**

### **Flow Cytometric Analysis of Gelatinase B (MMP-9) Activity using Immobilized Fluorescent Substrate on Microspheres**

#### **Reagents:**

Gelatin (300 Bloom) casein, fluorescein isothiocyanate (FITC), and 1,10-phenanthroline, were obtained from Sigma (St. Louis, MO). Bovine albumin was purchased from ICN Pharmaceuticals (Montreal, PQ, Canada). Purified gelatinase B was prepared as described (Masure *et al.*, (1993) *Eur. J. Biochem.* 218:129-141). The monoclonal antibody REGA-3G12 has been described by Paemen *et al.*, (1995) *Eur. J. Biochem.* (in press)). This monoclonal antibody binds to gelatinase B ( $K_d$ :  $2.1 \times 10^{-9}$ ) and inhibits the enzymatic activity.

#### Fluorescent Labeling of Substrates:

Casein and gelatin were dissolved at a final concentration of 2 mg/ml in carbonate buffer (pH 9.2). FITC (dissolved in DMSO at 5 mg/ml) was added to the indicated concentrations. Labeling was carried out for 24 h at 4°C. Free FITC molecules were removed by chromatography on PD-10 columns (Pharmacia, Uppsala, Sweden) using PBS, pH 7.4 as eluent buffer.

#### Protein Assay:

Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) to construct a standard curve.

#### Microsphere Coating:

Polystyrene microspheres of 15.5  $\mu\text{m}$  ( $\pm 1.919$ ) diameter (Polysciences, Warrington, PA) were incubated for 2 h at 37°C with FITC-conjugated substrates (1 mg/ml in PBS, pH 7.4) to allow noncovalent adsorption. This method was chosen for its simplicity and for the minimal conformational change it might induce (McHugh (1994) *supra*). The microspheres were then washed twice in phosphate buffer (pH 7.4) containing 0.5% BSA and 0.05% sodium azide (PBA). Microspheres were kept at 4°C in PBA ( $10^6$  beads/ml) in the dark and resuspended by gentle vortexing before use.

#### Gelatinase B digestion of FITC-Conjugated Substrates Immobilized on Microspheres:

The enzymatic reactions were carried out in a final volume of 100  $\mu$ l at 37°C for 16 hours in eppendorf tubes using RPMI medium (without FCS) as the reaction buffer. The samples contained 10  $\mu$ l gelatinase B-containing solutions, 10  $\mu$ l 10X solution of inhibitors or monoclonal antibodies, and 10  $\mu$ l FITC-labeled substrate-coated microspheres. The volume was completed with RPMI medium. The enzymatic reaction was stopped by adding 1 ml PBA, followed by two washes of the microspheres (1 min. at 8000 rpm). The pellets were then resuspended in 500  $\mu$ l PBA.

#### **Flow Cytometric Analysis:**

Samples were analyzed on a Coulter XL-MCL (Coulter Electronics, Hialeah, FL) using standard optics for detection of FITC fluorescence. A neutral density filter was used to lower the signal to the forward light scatter detector. Voltage of the photomultiplier tube used to detect FITC (FL1) was adjusted to allow visualization within the same histogram of the autofluorescence of the microspheres and the signal given by the microspheres coated with the FITC-labeled substrate. This voltage was kept constant for all analysis. Fluorescence was measured on a single parameter histogram using a log scale allowing for the discrimination of microspheres with a wide range of diameter. Between 1000 to 5000 events were analyzed for each histogram.

#### **Determination of Gelatinolytic Activity using FITC-labeled Substrate Immobilized on Microspheres:**

Figure 3 shows a typical one-parameter histogram illustrating the clear separation between the (auto)fluorescence of uncoated microspheres and the fluorescence of FITC-gelatin-coated microspheres. The two-parameter histogram with the forward-angle light scatter (FS) and side scatter (SS) on the x and y axes respectively was used to position the window on microspheres and to minimize interference with debris. Although the noise discriminator could be increased up to the levels of the microspheres, the present invention opted to display the "noise" so as to better monitor the quality of the samples. In most of the experiments, the

present invention obtained a signal-to-noise ratio near 500 between autofluorescent beads and those coated with the FITC-labeled substrate.

To determine the specificity of the enzymatic cleavage in immobilized FITC-conjugated gelatin on microspheres, the present invention involved the incubation of the FITC-gelatin- and control FITC-casein-coated microspheres with 200 ng purified gelatinase B from human neutrophil (Masure *et al.*, (1993) *supra*). The digestion temperature (37°C) and incubation time (16 hours) used were determined in preliminary experiments designed to establish the conditions necessary to achieve maximal sensitivity. Gelatinase B induced a 95% decrease of the fluorescent signal on gelatin-coated beads but not on the casein-coated beads (Figure 4).

To further confirm the specificity of the enzymatic reaction, a recently described gelatinase B-specific mouse monoclonal antibody with blocking activity was used (Paemen *et al.*, (1995) *supra*). The results of a representative experiment (Figure 5) show the dose-dependent blocking effect of inhibitory monoclonal antibody REGA-3G12. No significant differences were observed using mouse IgG control antibody (data not present).

#### **Inhibition of Gelatinase B Activity by Pharmacological Reagents:**

Gelatinase B activity has been shown to be inhibited by 1,10-phenanthroline, a specific inhibitor of zinc-dependent metalloproteinases. It is also inhibited by EDTA, since calcium ions are necessary to maintain a catalytically active conformation (Masure *et al.*, (1990) *Biochem. Biophys. Acta.* 1054:317-325). To determine whether the gelatinase B activity observed was sensitive to these agents, the activity of gelatinase B was measured in the presence of these inhibitors. Figure 6 shows that EDTA and phenanthroline inhibit more than 90% of gelatinase B activity. No inhibition was noted with sodium azide, but a significant inhibition was observed with DMSO.

#### **Sensitivity and Reproducibility of the Assay:**

The amount of FITC chemically linked to a protein can be controlled by varying the amount of FITC molecules added during conjugation. The FITC:protein (F:P) ration may theoretically interfere with the enzymatic activity of gelatinase B due to steric hindrance or conformational changes of the substrate; thus, five different stocks of microspheres were prepared, keeping the gelatin concentration constant during the coating, but varying the F:P ration on the gelatin molecules. The resulting stocks of FITC-gelatin-coated microspheres had a mean channel of fluorescence (MCF) ranging from 8 to 300 arbitrary units of fluorescence (Figure 7A). The data show that the sensitivity of the assay was not affected by the F:P ration (Figures 7B and 7C). Therefore, the FITC does not interfere with enzymatic activity, either through the hydrophobicity generated by the labeling, or through steric hindrance around the cleavage sites. Using an F:P ration that approaches the plateau of maximal MCF therefore generates a higher dynamic range, and precludes the possibility of quenching between FITC molecules.

Although it was expected that the FITC might interfere with the coating through the hydrophobicity generated by the labeling, the F:P ration did not affect the sensitivity of the assay.

The linear range of the assay extended from 1 to 200 ng of gelatinase B. The variation between samples was tested and the results obtained was highly reproducible. For example, in most experiments, the variation was between 0.5 to 1% among samples (Figures 5 and 7). This high homogeneity among samples was also evident when the same samples were measured with different protocols of acquisition (Table 1). In the present invention, the MCF of a microsphere sample with approximately 50% FITC-gelatin degraded on its surface was measured; the sample was then run in different conditions of acquisition. Varying the flow rate and the total number of events analyzed had no significant impact on the MCF. This is consistent with previous observations using microspheres coated with capture reagents in flow microsphere immunoassays (McHugh (1994) *supra*).



Table 1.		
Data Reproducibility		
Number of Events	Flow Rate	MCF <sup>1</sup> (s.d.)
10,000	500-700 events/s	69.9 (0.2)
1,000	500-700 events/s	70.1 (0.4)
10,000	50 events/s	ND <sup>2</sup>
1,000	50 events/s	69.7 (0.8)

<sup>1</sup>Mean Channel of Fluorescence: average of triplicates.

<sup>2</sup>Not doable since the beads pellet at the bottom of the tube.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. Such changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims.

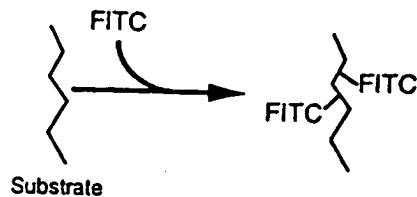
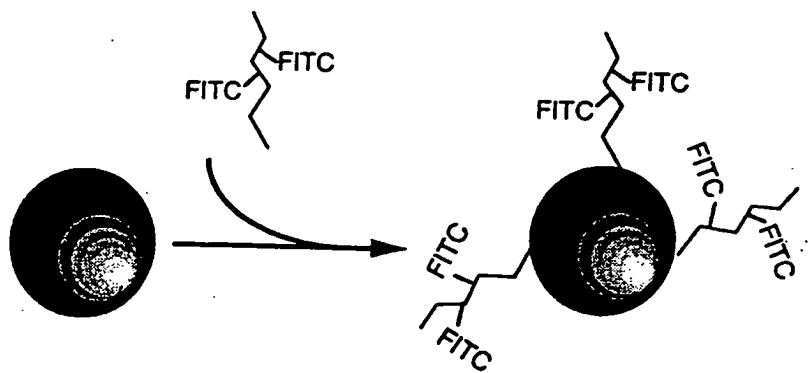
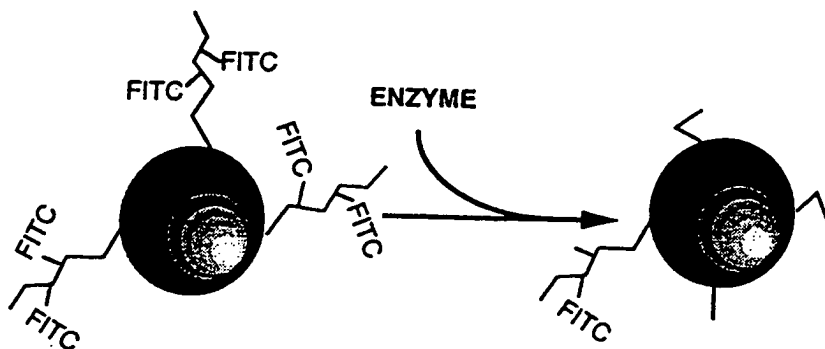
**STEP I: Fluoresceination of substrates:****STEP II: Microsphere coating.****STEP III: Enzymatic digestion.**

Figure 1 of 7

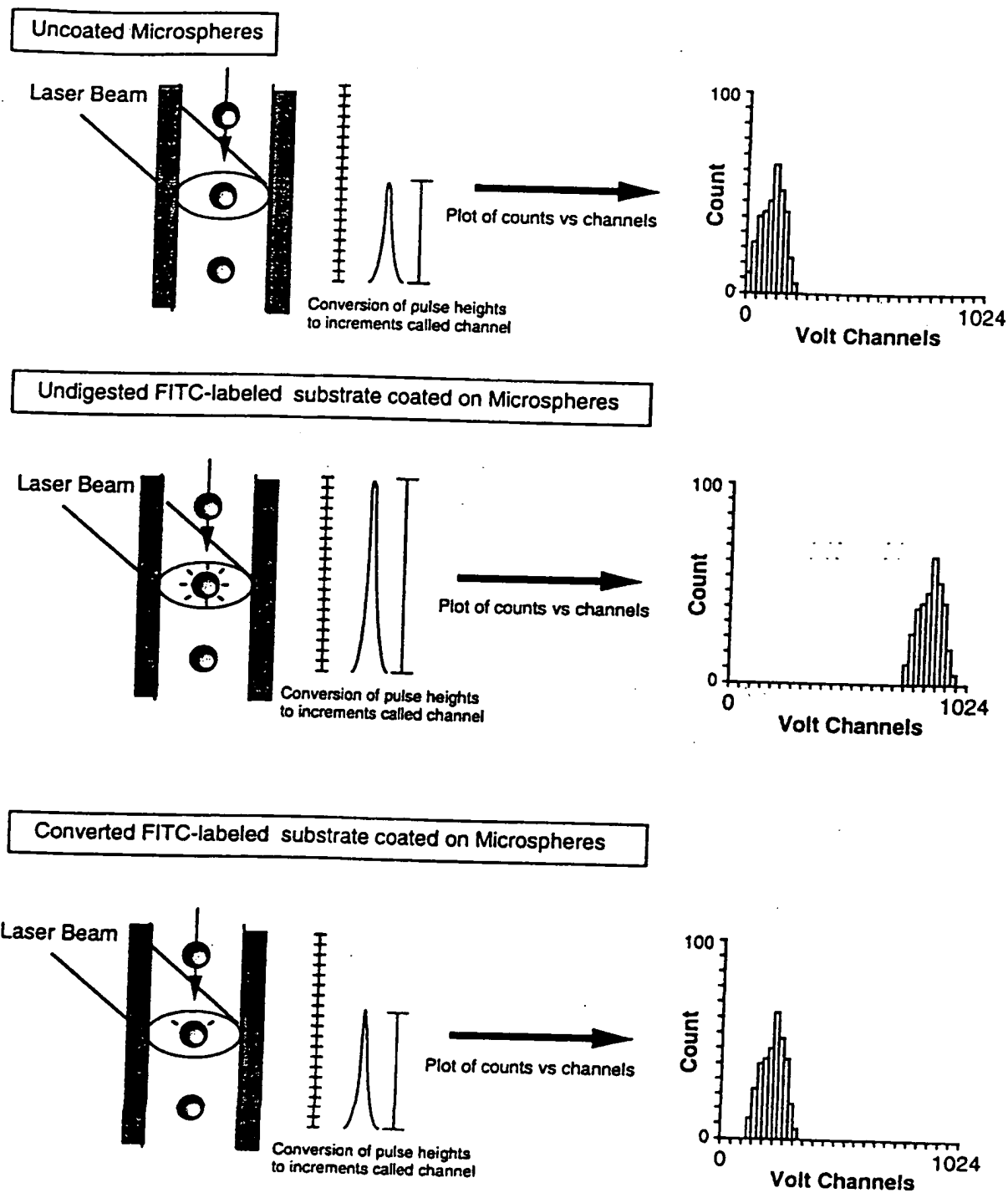


Figure 2 of 7

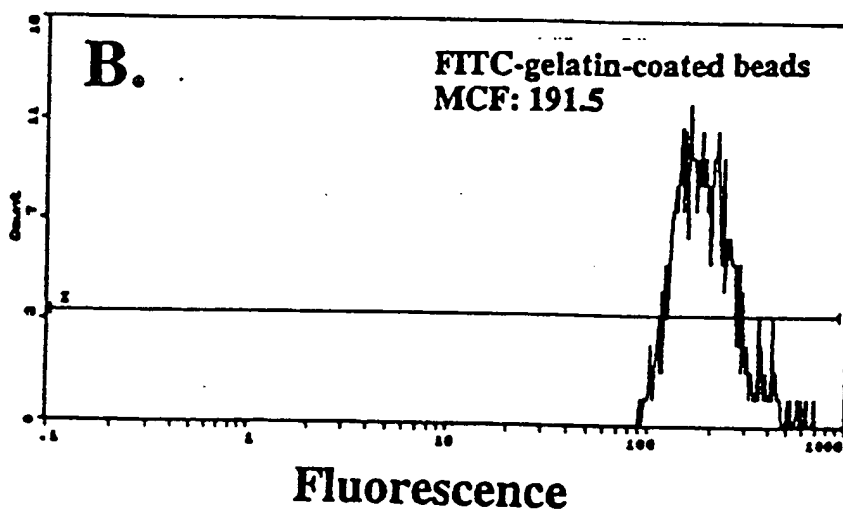
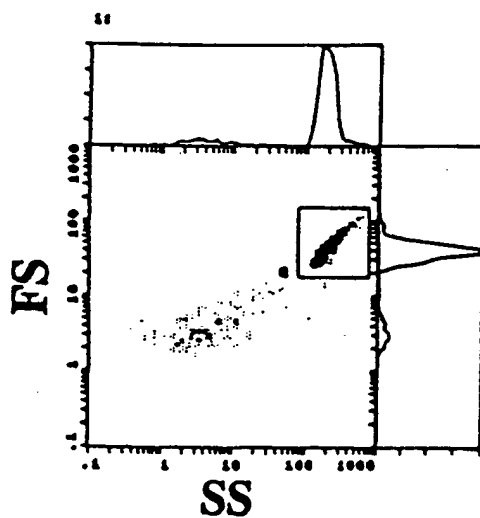
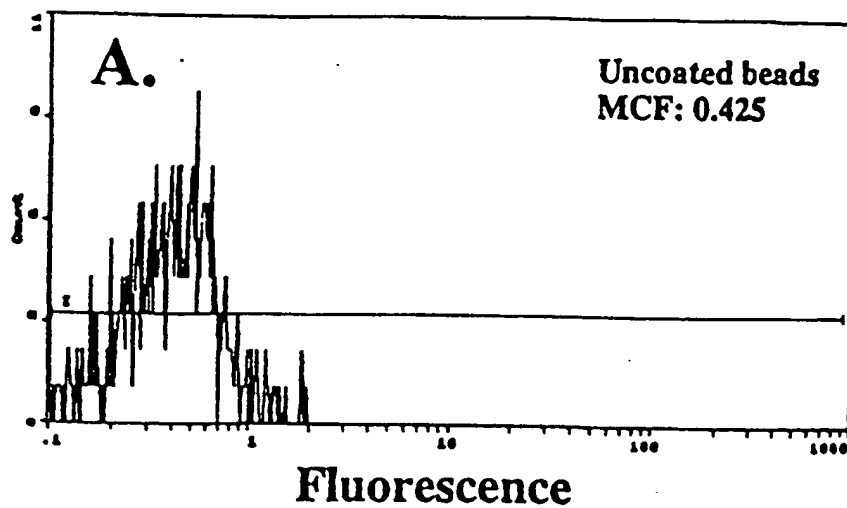
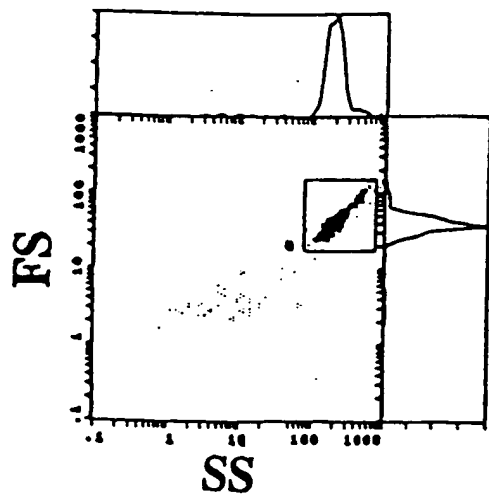


Figure 3 of 7

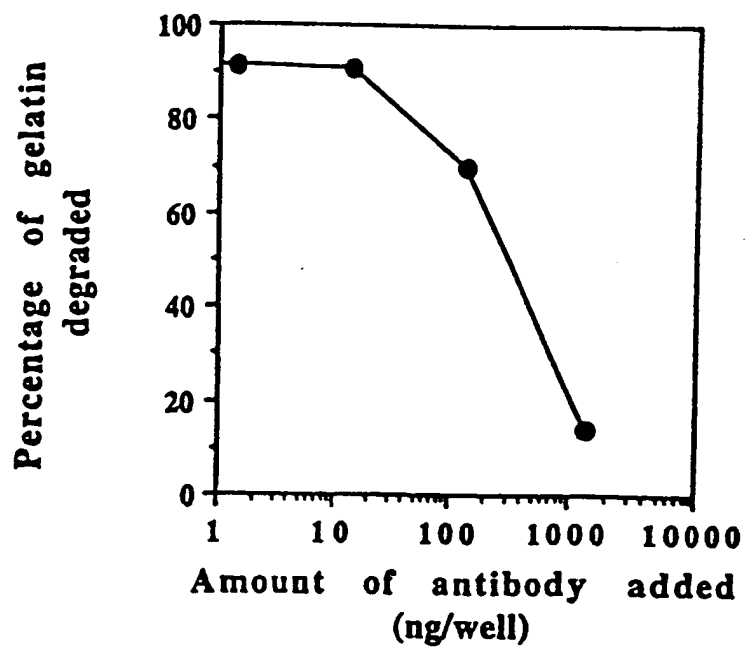


Figure 4 of 7

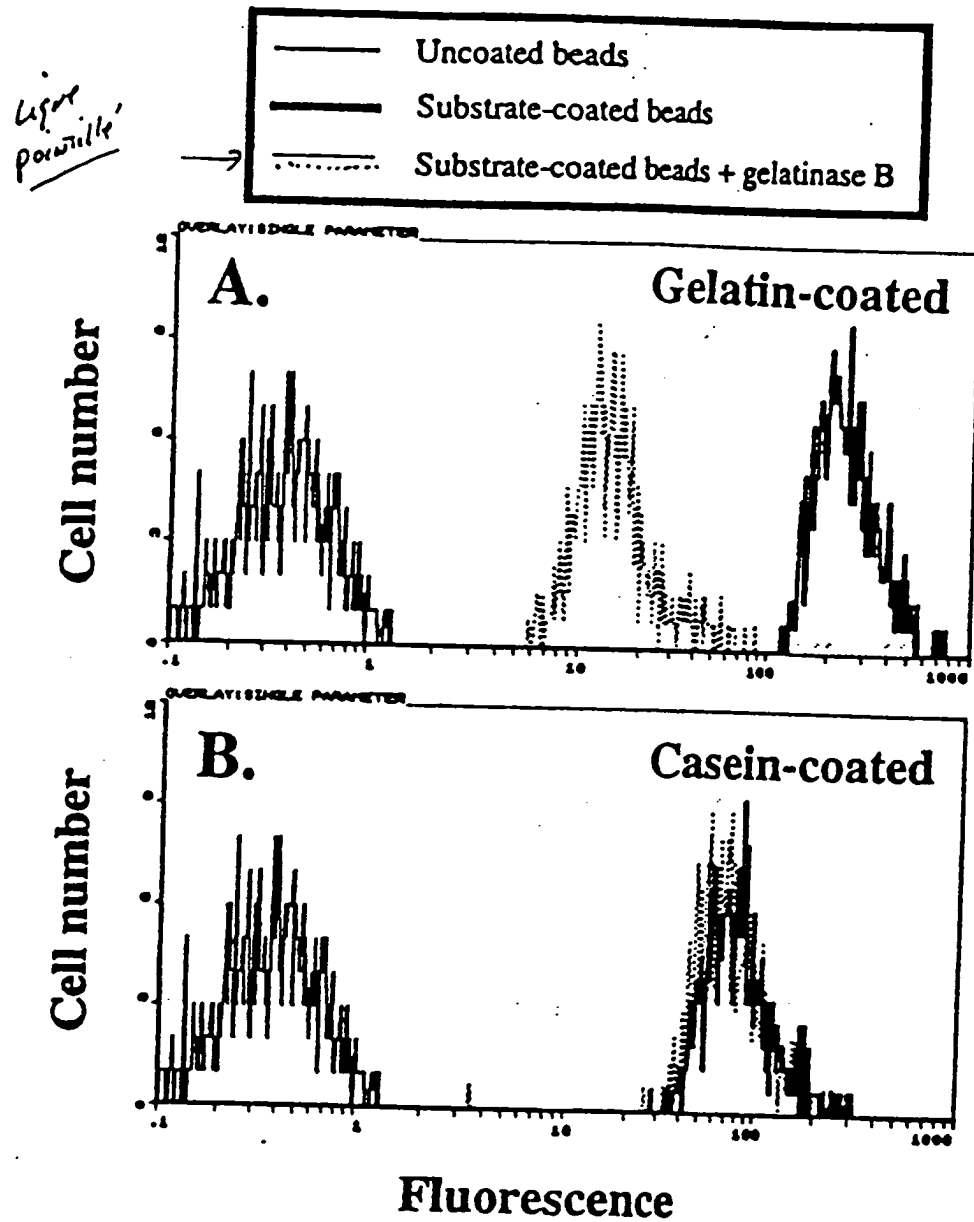


Figure 5 of 7

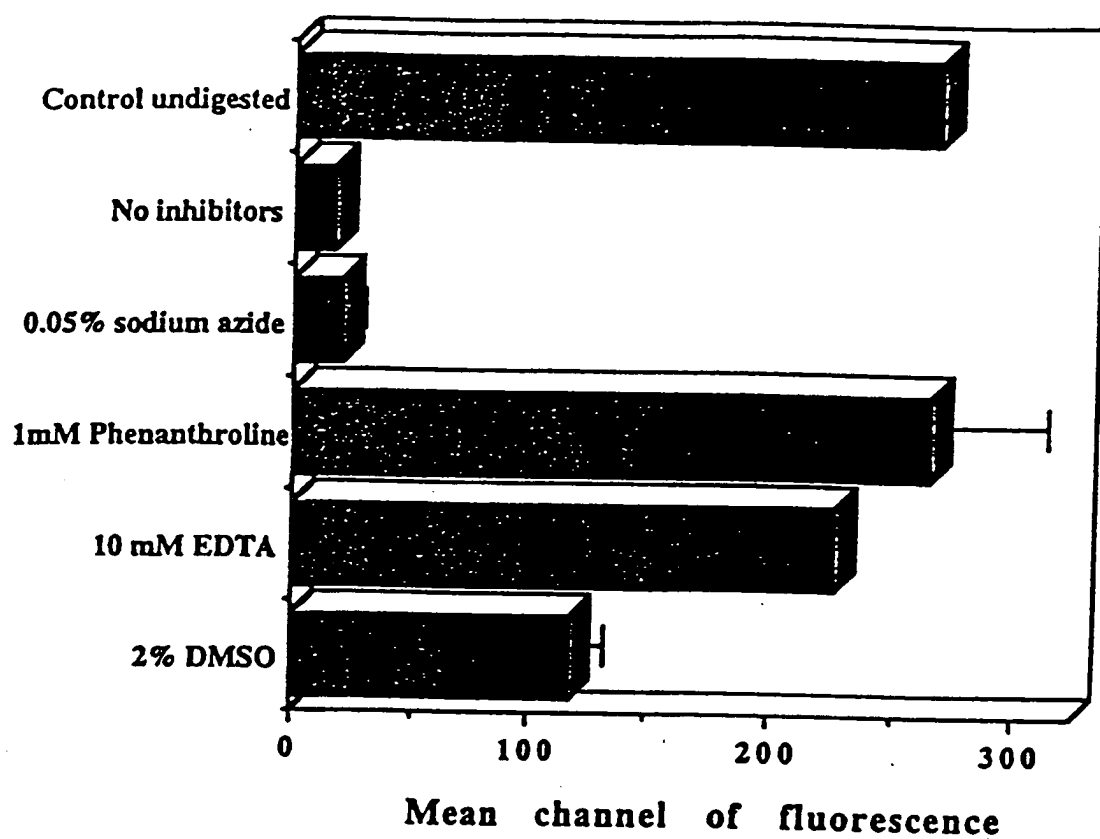


Figure 6 of 7

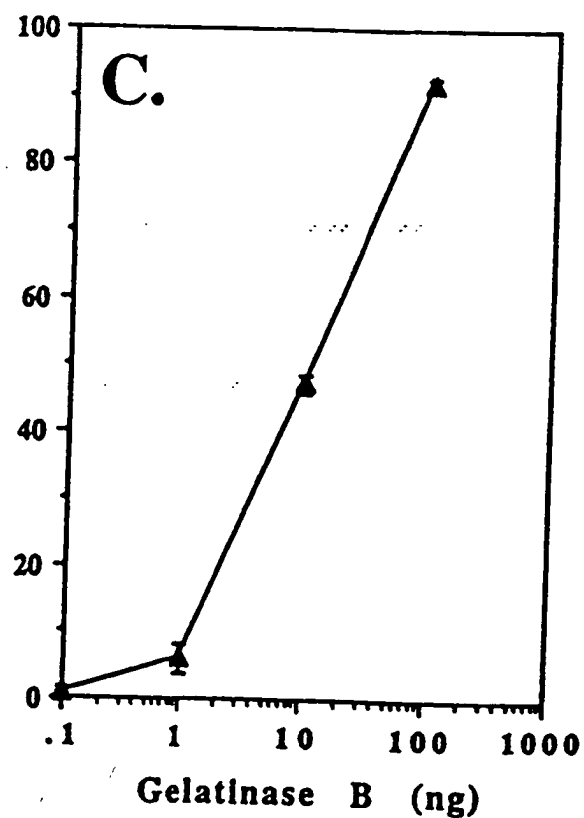
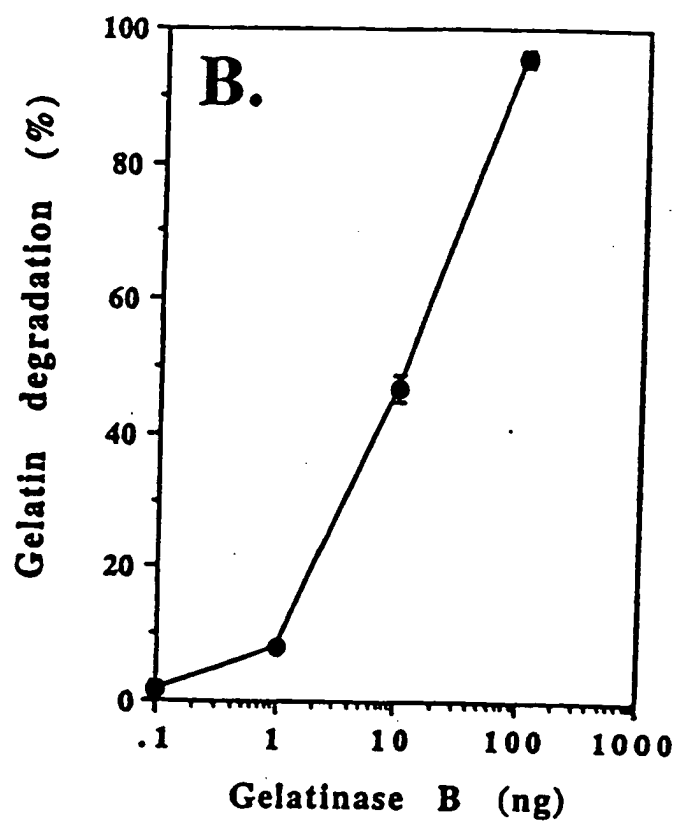
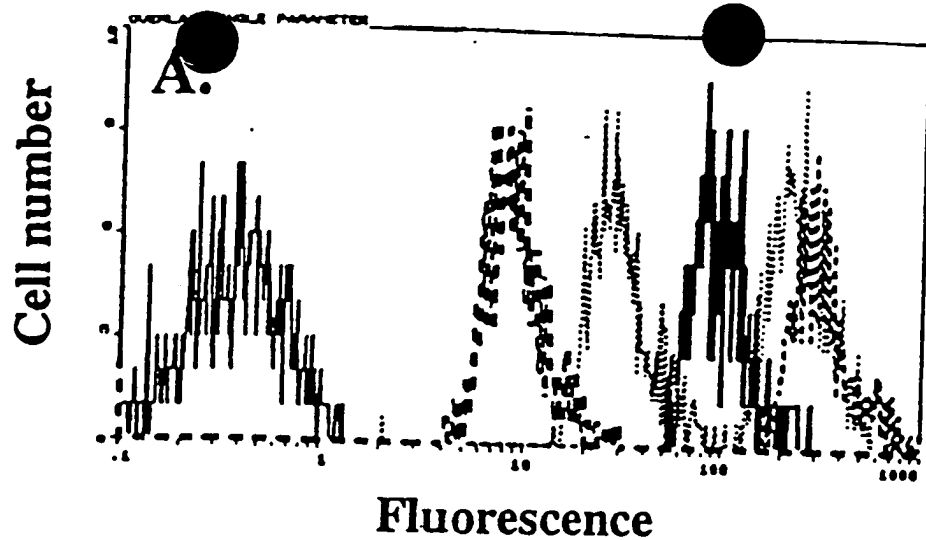


Figure 7 of 7